

### REMARKS

Claim 8 has been amended. Claims 1 and 4-9 are now pending in this application. Support for the amendments is found in the existing claims and the specification as discussed below. Accordingly, the amendments do not constitute the addition of new matter. Applicant respectfully requests the entry of the amendments and reconsideration of the application in view of the amendments and the following remarks.

#### Drawings

Applicants submit herewith a set of replacement drawings in black and white.

#### Rejection under 35 U.S.C. § 112, first paragraph

Claims 1 and 4-9 are rejected under 35 U.S.C. § 112, first paragraph as lacking enablement.

According to the examiner, it cannot be predicted from the patent specification if therapeutic levels of gene expression will be obtained after direct or indirect administration of vectors. In addition, the examiner considers that it is unpredictable to extend results of animal systems to humans. In response, Applicants submit Attachments A and B herewith which are two publications by the present inventors. Applicants assert that, in addition to the specification of the present application, these documents provide further enabling teachings with respect to the transfection of human and animal cells with an expression vector comprising the claimed polynucleotides.

In Biochem. Biophys. Res. Commun. 292, 848-854 (2002) (submitted herewith as Attachment A), the inventors have successfully transfected simian COS-7 cells with an OC-3 expression vector and shown the correct translation of the gene in said cells (Figure 2). Furthermore, Human Embryonic Kidney 293 cells (HEK-293) were successfully transfected with an OC-3 expression vector and the produced OC-3 protein was able to stimulate the transcription of target genes (Figure 3; page 853, col. 1, first full paragraph).

In Biochem. Biophys. Res. Commun. 285, 1200-1205 (2001) (submitted herewith as Attachment B), the inventors have shown that the transcription factor OC-2 controls the Microphthalmia-associated transcription factor (MITF) gene (Figure 4, for example), which is essential for melanocytes differentiation. MITF mutations are associated with some cases of

Waardenburg syndrome (page 1203, col. 1, line 3 to col. 2, end of first para.). It was shown that overexpression of OC-2 in transfected cervix carcinoma cells stimulates MITF promoter activity.

The data provided in Attachments A and B provide evidence of high levels of expression for the claimed polynucleotides encoding peptides of the ONECUT family, sufficient to provide the disclosed benefits following administration as claimed (claims 7-9). The Examiner is reminded that clinical data is not required to meet the enablement requirement of 35 U.S.C. § 112, first paragraph. The present specification describes methods by which polynucleotides of the claimed invention may be administered to a patient or to cell lines of a patient via *ex vivo* treatment (present specification, page 6, lines 24-32). The present specification describes methods for using the claimed pharmaceutical compositions in cell therapy, *in vivo* and *ex vivo* (present specification, page 7, lines 7-13). The specification describes the components of the pharmaceutical composition (present specification, page 7, lines 14-34). Specifically, the present specification describes a method of implanting cells transformed with either HNF-6 or OC-2 into a diabetic animal (see page 13, line 8 to page 14, line 17). Thus, the present specification provides the necessary teaching for the practice of the claimed invention. The two Attachments evidence that the claimed invention operates as predicted by the specification.

Attachment A submitted herewith demonstrates that the claimed transcription factors such as OC-3 are produced in transformed cells at a level sufficient to stimulate the desired transcription of target genes. Attachment B shows that the claimed elements (e.g. OC-2) function in the manner predicted by the specification, that is, OC-2 effects transcription of the MITF gene. Applicants maintain that the specification teaches one skilled in the art how to make and use the claimed invention and that Attachments A & B submitted herewith would indicate to the skilled art worker that the claimed pharmaceutical compositions would be effective in the treatment of disease conditions in the manner described in the present specification, such as Waardenburg syndrome type 2 as discussed in Attachment B (see especially Attachment B at page 1203, col. 1, line 3 to col. 2, end of first para.).

In view of Applicants' arguments and Attachments A & B, reconsideration and withdrawal of the above ground of rejection is respectfully requested.

**Claim objections**

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The Examiner objects to claim 8 as depending from cancelled claims 2 and 3. In response, claim 8 was amended to depend from claim 7 with the Preliminary Amendment filed on February 20, 2001.

**Rejection under 35 U.S.C. § 112, second paragraph**

Claim 8 is rejected under 35 U.S.C. § 112, second paragraph as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

The Examiner asserts that claim 8 is vague and indefinite as it is not clear how a cell can be transformed by treating the cell with an OC-2 polypeptide or a cell line transformed with a vector comprising an OC-2 polypeptide.

In response, Applicants have amended claim 8 to refer to the pharmaceutical composition as comprising either a polynucleotide encoding HNF-6, OC-2 or OC-3 or a vector comprising said polynucleotide. Support for the amendment is found in the original claims.

In view of Applicants' amendment, reconsideration and withdrawal of this ground of rejection is respectfully requested.

**Rejection under 35 U.S.C. § 102(a)**

Claims 1, 3, and 5 are rejected under 35 U.S.C. § 102(a) as being anticipated by Jacquemin, et al. (1999) *J. Biol. Chem.* 274: 2665-2671.

This ground of rejection is overcome by the certified English translation of Applicants' priority document submitted herewith as Attachment C. This ground of rejection may now be properly withdrawn.

**CONCLUSION**

In view of Applicants' amendments to the claims and the foregoing Remarks, it is respectfully submitted that the present application is in condition for allowance. Should the Examiner have any remaining concerns which might prevent the prompt allowance of the application, the Examiner is respectfully invited to contact the undersigned at the telephone number appearing below.

Appl. No. : 09/763,535  
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Please charge any additional fees, including any fees for additional extension of time, or credit overpayment to Deposit Account No. 11-1410.

Respectfully submitted,

KNOBBE, MARTENS, OLSON & BEAR, LLP

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## ATTACHMENT A

## OC-3, a Novel Mammalian Member of the ONECUT Class of Transcription Factors

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Transcription factors of the ONECUT class possess a single cut domain and a divergent homeodomain. They regulate gene networks by controlling the expression of other transcription factors and they play an important role in cell differentiation and metabolism. We identified earlier in mammals HNF-6 (ONECUT-1), the founding member of the class, and ONECUT-2 (OC-2). We have now characterized in the mouse a third ONECUT member, which we call OC-3. Its gene is located on chromosome 10. The sequence of OC-3 (490 residues) displays 51% amino acid identity with HNF-6 and 50% with OC-2. OC-3 has a DNA-binding specificity similar to that of HNF-6 and it is a stimulator of gene transcription. OC-3 mRNA is found in brain, stomach, and upper intestine in the adult and embryonic mouse. Our earlier work on HNF-6 and the expression patterns of the three mammalian ONECUT genes suggest that they all participate to the control of organ development from the foregut and midgut endoderm.

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**Key Words:** ONECUT; HNF-6; OC-2; homeodomain proteins; transcription factors; endoderm; gut.

Transcription factors of the ONECUT class, whose prototype is mammalian Hepatocyte Nuclear Factor-6 (HNF-6) (1), possess a single cut domain (66 residues) and a divergent homeodomain characterized by the presence of a phenylalanine instead of a tryptophan at position 48 and of a methionine at position 50 of the 60-residue homeodomain (2). In HNF-6 these two domains are involved in both DNA binding and transactivation (2, 3). ONECUT proteins have been identified not only in rats (1), mice (4) and humans (5, 6), but also in the nematode *Caenorhabditis elegans* (2), the ascid-

ian *Halocynthia roretzi* (7), the fly *Drosophila melanogaster* (8), and the zebrafish *Danio rerio* (9). This conservation during evolution suggests that the ONECUT transcription factors control basic cellular processes. Work on HNF-6 has shown that it stimulates the expression of transcription factors which in turn control a number of genes (2, 4, 10–12). In this way, HNF-6 regulates complex genetic programs and indeed studies on *hnf6* knockout mice showed that it plays an important role in glucose metabolism and in embryonic development. For instance, HNF-6 controls the specification of the pancreas from the endoderm by triggering expression of the transcription factor Pdx-1 (our unpublished data), the development of the endocrine pancreas at the precursor stage by stimulating the gene coding for the transcription factor Ngn-3 (11), and the development of the biliary tract by stimulating the gene coding for the transcription factor HNF-1 $\beta$  (12). Such observations, as well as data on *Drosophila* (8) and *Halocynthia* (7) ONECUT proteins, show that the ONECUT transcription factors control the differentiation of pluripotent precursor cells. The identification of additional ONECUT proteins in mammals and of their tissue pattern of expression is therefore of great interest. Besides HNF-6, we identified a second mammalian ONECUT protein which we called ONECUT-2 (OC-2) (5). We showed that OC-2 controls the gene coding for the melanocyte differentiation factor MITF (*Microphthalmia*-associated transcription factor) (13). We now describe in the mouse the third, and most likely last, mammalian member of this class, which we call OC-3, and we characterize its properties.

### MATERIALS AND METHODS

**Oligonucleotides and antibodies.** The polymerase chain reaction (PCR) primers were as follows: HOM1, 5'-CKNSYRTTCATRAA-RAA-3'; HOM2, 5'-CCYMGRTYSGYSTTYAC-3'; moc3.1, 5'-GCTGAAGCGCTACAGCAT-3'; moc3.4, 5'-GATCAGGTACCTCAGGCCTGGAGAAGGTG-3'; moc3.16, 5'-ATGTCGGCGCTGCGCCTG-3'. The antiserum UC156 was obtained from a rabbit immunized with a

The nucleotide sequence of mOC-3 cDNA has been submitted to GenBank/EBI Data Bank under Accession No. AY080897.

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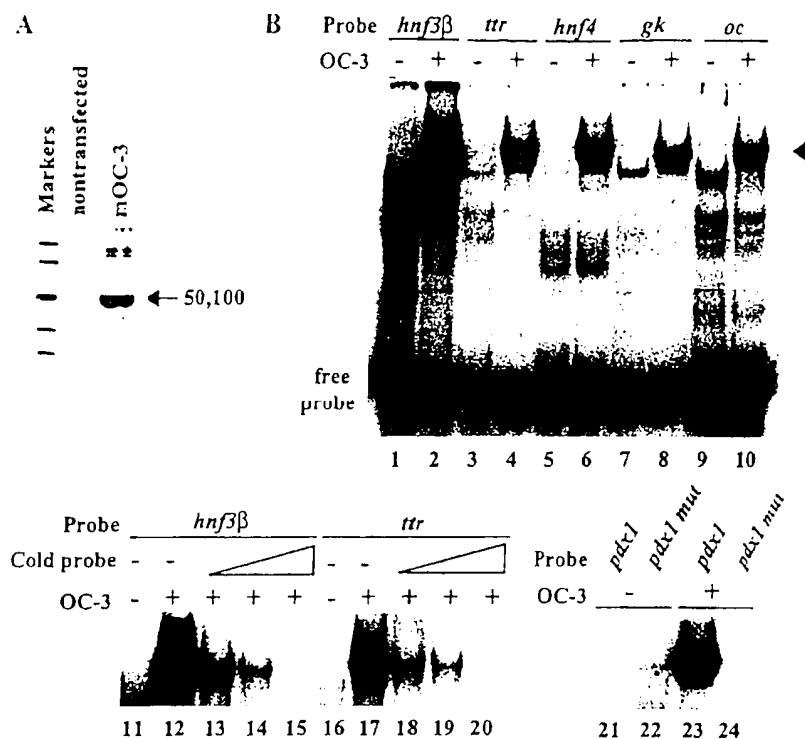


FIG. 2. Expression and DNA binding of OC-3. (A) OC-3 mRNA is translated in a protein of the expected mass. mOC-3 expressed in COS-7 cells was detected by immunoblotting with the UC106 anti-peptide antibody after separation of proteins by PAGE. (B) OC-3 binds to known HNF-6 binding sites. Extracts from COS-7 cells transfected (+) or not (-) with the mOC-3 expression vector were tested by EMSA for binding to the probes indicated above the lanes and defined in Table 1. The arrowhead points to the specific OC-3/DNA complexes. Excess cold probe was 5-, 10-, or 50-fold in lanes 13-15 and 18-20.

synthetic peptide corresponding to residues 483 to 490 of mouse OC-3.

**Cloning of the mouse *oc3* gene.** A 110-bp OC-3 probe was synthesized using the degenerate PCR primers HOM1 and HOM2 and mouse genomic DNA as template. This probe was used to screen the RPCI21 PAC Library (Roswell Park Cancer Institute) constructed in the pPAC4 vector and containing inserts of about 150 kb. Clones (130,000) were hybridized at 42°C in 6× SSC, 50% formamide and washed at 60°C in 2× SSC. After overnight exposure on Kodak Xomat autoradiographic film a single positive clone was detected. The two exonic regions of the *oc3* gene were excised from this clone using *EcoRI*, which yielded a fragment of 7.5 kb containing exon 1, and *HindIII*, which yielded a fragment of 4.3 kb containing exon 2. These fragments were subcloned in pBlueScript and sequenced on both strands.

**Expression vectors and reporter constructs.** Because the GC content of the 5' region of OC-3 mRNA is very high, we failed to obtain a fully coding mOC-3 cDNA by reverse transcriptase (RT)-PCR on mouse RNA. We therefore associated two restriction fragments. The 5' one was obtained by digesting the genomic subclone containing exon 1 with *SgrAI* and *Eco47III*. This fragment of 1160 bp, 170 of which are upstream of the ATG, contains exon 1 without its last 185 bp. The other fragment was obtained by RT-PCR on brain RNA from a mouse at embryonic day (E) 14.5 with primer *oc3.1*, which is located in exon 1, and primer *oc3.4*, which overlaps the stop codon. This fragment was digested with *Eco47III* and *Asp718* to produce a

480-bp fragment containing the last 185 bp of exon 1 and the coding portion of exon 2. The 1160- and 480-bp fragments were ligated in the *XmaI* and *Asp718* sites of the pXJ40 eukaryotic expression vector (14) to obtain pXJ40-OC3, which was verified by restriction and sequencing. The pHNF-3β(6×)-TATA-luc and pTTR(6×)-TATA-luc reporters have been described (2). The internal control vector pRL138 contains the rat *pA2* promoter (-138 to -56) cloned in the vector pRLnull (Promega) to provide a constitutive expression of Renilla luciferase (2).

**Cell culture, transfection, and extracts.** COS-7 cells were transfected for 6 h with 5 μg of pXJ40-OC3 using LipofectAMINE PLUS (Life technologies, Inc.) in Dulbecco's modified Eagle medium (DMEM) without serum. After 24 h, the cells were washed with phosphate-buffered saline and lysed in 150 μl of 20 mM Hepes (pH 7.5), 10% glycerol, 400 mM KCl, 0.4% Triton X-100, 10 mM EGTA, 5 mM EDTA, 1 mM dithiothreitol, 0.4 mM phenylmethylsulfonyl fluoride, 20 mg/L pepstatin, 10 mg/L leupeptin, and 10 mg/L aprotinin. The lysates were briefly sonicated and centrifuged and the supernatants were collected. HEK-293 cells were grown in DMEM supplemented with 10% fetal bovine serum. Cells (7.5 × 10<sup>4</sup> cells/well on 24-well plates) were transfected in medium without serum using DOTAP Liposomal Transfection Reagent (Roche), with 400 ng of pHNF-3β(6×)-TATA-luc or pTTR(6×)-TATA-luc, 40 ng of pXJ40-OC3 or pXJ40, and 30 ng of pRL138 as internal control. After 6 h, the medium was removed and the cells were incubated for 24 h in DMEM plus 10% serum before measuring luciferase activities with



TABLE 1  
DNA-Binding Specificities of the Three Mammalian OneCut Transcription Factors

| Target gene                  | Oligonucleotide sequence <sup>a</sup> | Relative binding efficiency <sup>a</sup> |       |      |
|------------------------------|---------------------------------------|--|-------|------|
|                              |                                       | OC-3                                     | HNF-6 | OC-2 |
| <i>hnf3β</i>                 | GAAAAA <b>AAATCAATAT</b> CGGGCCT      | +++                                      | +++   | +-   |
| Transthyretin ( <i>ttr</i> ) | GTCTGCT <b>TAAGTCAATAAT</b> CAGAAT    | +  | +++   | +    |
| <i>hnf4</i>                  | AGGATAGAA <b>GTCAATGAT</b> CTGGGA     | ++                                       | +     | -    |
| Glucokinase ( <i>gk</i> )    | GGGAAAG <b>TGATCAAT</b> CGTGCAAG      | +  | +++   | ++   |
| <i>mitf</i> ( <i>oc</i> )    | AAAAA <b>TAATCAACAT</b> TTAA          | +  | +++   | ++   |
| <i>pdx1</i> (distal site)    | AGGCAT <b>GTAATCAATAA</b> TAACAT      | +  | ++    | ++   |
| <i>pdx1</i> mutated          | AGGCAT <b>TAACAAATAA</b> TAACAT       | -  | -     | -    |

<sup>a</sup> These sequences, used for the EMSA experiments shown in Fig. 2B, originate from the promoters of the genes listed. The part in bold corresponds to the 10-bp consensus defined earlier for HNF-6 (2).

<sup>a</sup> Data taken from the present paper and references cited therein and from our unpublished experiments with HNF-6 and OC-2.

the Dual-Luciferase kit (Promega) and a Lumac luminometer. Relative activities were expressed as the ratio of firefly luciferase to *Renilla* luciferase.

**Electrophoresis.** For polyacrylamide gel electrophoresis (PAGE) and immunoblotting, lysates of COS-7 cells transiently transfected with pXJ40-OC3 were prepared. Total protein (30 µg) was loaded on a 10% polyacrylamide gel and separated by SDS-PAGE. After electrotransfer, the membrane was probed with the polyclonal rabbit OC-3 antiserum. For electrophoretic mobility shift assays (EMSA), COS-7 lysates (5 µl) were incubated on ice for 20 min in a final volume of 20 µl containing 10 mM HEPES (pH 7), 1 mM dithiothreitol, 1 mM MgCl<sub>2</sub>, 0.5 mM EDTA, 50 mM KCl, 10% glycerol, 4 µg of poly(dI-dC) and the <sup>32</sup>P-labeled probe (30,000 cpm). The samples were loaded on a 6% polyacrylamide gel in 0.25× Tris borate/EDTA buffer and electrophoresed at 200 V.

**Reverse-transcription PCR (RT-PCR).** To determine the distribution of OC-3 mRNA, RT was performed with 1 µg of total RNA isolated from mouse organs using Moloney murine leukemia virus reverse transcriptase and random hexamers. OC-3 cDNA was amplified by PCR performed on 1/25 of the RT reaction with primers *moc3.4* (in exon 2) and *moc3.16* (in exon 1) to yield a fragment of 322 bp which was detected by ethidium bromide staining after electrophoresis in 1.4% agarose gel. The integrity of the RNA preparation was tested by amplification of a 192-bp TBP cDNA fragment (11).

## RESULTS AND DISCUSSION

**Identification and cloning of OC-3.** To search for ONECUT factors related to HNF-6 and OC-2 we first screened the human genome data banks using the BLAST algorithm. We looked for open reading frames (ORF) coding for proteins containing a single cut domain and an homeodomain whose amino acids at positions 48 and 50 are a phenylalanine and a methionine, respectively. We found on chromosome 19 a sequence that matched these criteria and differed from the sequence of HNF-6 (OC-1) and OC-2 (5). We therefore called it OC-3. This sequence has the same organization as the HNF-6 and OC-2 genes, with a first coding exon that includes the cut box and a second coding exon that includes the homeobox (5, 15).

We decided to clone OC-3 from mouse (*mOC-3*), the most convenient species for further gene function anal-

ysis in mammals. Degenerate oligonucleotides localized in the homeobox were synthesized on the basis of conserved ONECUT sequences. A PCR on mouse genomic DNA with these primers yielded a 110-bp fragment whose sequence differed from those of *hnf6* and *oc2*. A comparison of the ORF coded by this fragment with human sequences strongly suggested that it belonged to the *moc3* gene. This 110-bp fragment was used to screen a mouse genomic DNA library and a single positive clone was obtained. In the meantime we discovered in the GenBank a draft sequence (Accession No. AC073691) of the *moc3* gene. This allowed us to confirm that the 110-bp fragment was part of the *moc3* gene and to obtain information for subcloning the two coding exons of our positive clone in view of accurate sequencing. This yielded an ORF of 490 amino acids (Fig. 1). The initiator ATG was in a perfect Kozak consensus and it was preceded by a stop codon 27 nucleotides upstream. The alignment of this sequence

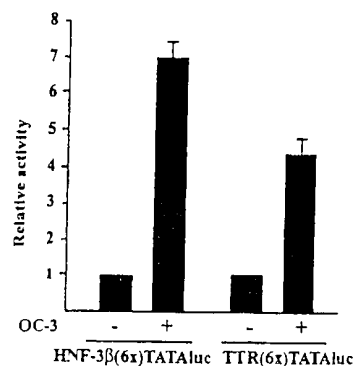


FIG. 3. OC-3 is a stimulatory transcription factor. HEK-293 cells were transfected with the *mOC-3* expression vector (+) or the empty vector (-) and with the reporter constructs indicated. Relative promoter activities were normalized for the value measured in absence of the OC-3 expression vector (mean ± SE, n = 4).

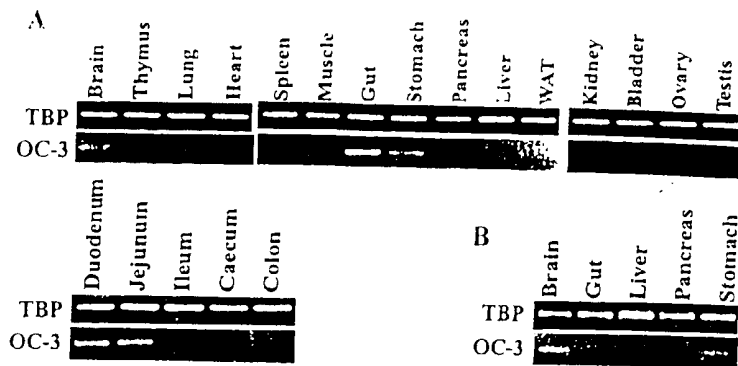


FIG. 4. OC-3 is expressed in the brain, stomach, and proximal intestine. OC-3 mRNA was detected by RT-PCR on RNA from the indicated organs of adult (A) or embryonic (B) mouse. TBP, TATA-binding protein mRNA detected as a control. WAT, white adipose tissue.

with that of hOC-3 showed an overall identity of 90% (Fig. 1), with 100% identity in the cut domain and only one different residue in the homeodomain. The identities between OC-3 and HNF-6 were of 97% for the cut domain and 83% for the homeodomain. The LSDLL motif in the cut domain shown earlier to be crucial for the interaction of HNF-6 with coactivators (3) was conserved in OC-3. Like in mHNF-6, the linker between the two domains had 27 residues, 20 of which were identical in OC-3 and HNF-6. No evidence was found, by RT-PCR on RNA from mouse organs (see below) and by data bank searches, for an 78-bp exon between exon 1 and exon 2 in the *oc3* gene, as is the case for the *mhnf6* gene.

In HNF-6, a serine/threonine/proline-rich region (STP box) located in the N-terminal moiety had been identified as participating to the stimulatory effect on transcription (3). This box was present in OC-3 (76% of amino acid sequence identity with that of HNF-6), but it contained no serine residue. We therefore renamed this region the TP box. The polyhistidine tract found in the N-terminal moiety and the polyserine tract found in the C-terminus of HNF-6 were absent from the OC-3 sequence. However, there were five threonine residues in the C-terminus of OC-3. Finally, the OC-3 sequence contained two polypoline and three polyglycine tracts (Fig. 1).

In the mouse, the *hnf6* gene has been assigned to chromosome 9 (4). By screening the mouse genome maps (<http://mouse.ensembl.org>) with the sequence of the *oc3* gene, we assigned this gene to the C1C2 region of chromosome 10.

OC-3 has DNA-binding properties similar to those of HNF-6. The high amino acid sequence similarity between the cut domain and homeodomain of HNF-6 and those of OC-3 suggested that the two proteins recognize similar DNA sequences. To test this, we constructed an OC-3 expression vector (see Materials and

Methods) and verified that it was translated properly. To do so, we transfected COS-7 cells with the expression vector, separated cell lysate proteins by PAGE and detected OC-3 by immunoblotting with the anti-OC-3 antibody. A single band was obtained whose  $M_r$  (50,100) fitted the calculated molecular mass of 49,646 Da (Fig. 2A). EMSA was performed on six different sequences known to bind HNF-6 (Table 1). Five of these sequences contained the typical TCAAT core conserved in all HNF-6 target sequences defined so far in gene promoters (2). They were chosen because they differ in the sequence flanking this conserved core sequence. The sixth sequence tested (probe *oc*) contained a divergent TCAAC core which was shown earlier to bind OC-2 (13) and HNF-6 (our unpublished data). As illustrated in Fig. 2B, lanes 1–10 and 21–23, mOC-3 bound to the six probes tested. The binding was specific, as demonstrated by competition with excess cold

TABLE 2  
Pattern of Expression of the Three Mammalian One-cut Genes in Adult Mouse Organs, by RT-PCR Analysis

| Organ                | HNF-6 | OC-2 | OC-3 |
|----------------------|-------|------|------|
| Brain                | ++    | ++   | ++   |
| Thymus               | -     | -    | -    |
| Lung                 | -     | -    | -    |
| Heart                | -     | -    | -    |
| Spleen               | -     | -    | -    |
| Stomach              | -     | ++   | ++   |
| Gut                  | -     | ++   | ++   |
| Liver                | ++    | ++   | ++   |
| Pancreas             | ++    | -    | -    |
| White adipose tissue | -     | -    | -    |
| Kidney               | -     | -    | -    |
| Urinary bladder      | -     | -    | -    |
| Ovary                | -     | -    | -    |
| Testis               | +     | -    | -    |

probe (Fig. 2B, lanes 11–20) and by lack of binding to the mutated *pdx1* oligonucleotide (lanes 21–24), which binds neither HNF-6 nor OC-2. These data, together with those in Table 1, show that the binding specificities of OC-3 and HNF-6 are similar, but differ slightly from those of OC-2, which does not recognize the *hnf4* probe (5).

*OC-3 is a stimulator of transcription.* Although HNF-6 and OC-2 stimulate transcription of target genes, the *Halocynthia* ONECUT protein reportedly represses transcription (7). As the sequence of OC-3 differs from that of HNF-6 outside the cut-homeodomain region, we tested whether OC-3 stimulates or represses gene transcription. HEK-293 cells were co-transfected with the mOC-3 expression vector and a reporter construct containing the luciferase gene driven by a TATA box linked to the multimerized ONECUT binding site found in the *hnf3 $\beta$*  gene or in the *trans-thyretin* gene. As shown in Fig. 3, OC-3 stimulated transcription of the two constructs. We concluded that, like the two other mammalian ONECUT proteins, OC-3 behaves as a stimulatory transcription factor.

*Expression pattern of the oc3 gene.* To determine the distribution of OC-3 mRNA, we screened RNA from fifteen adult mouse organs by RT-PCR. As a control of RNA integrity, TBP mRNA was amplified from the same RNA preparations. It was found that OC-3 mRNA is present only in the brain, stomach and gut (Fig. 4A). To analyze its distribution along the gut, we extracted RNA from different segments. OC-3 mRNA was found in the duodenum and jejunum, but not in the more distal parts (Fig. 4A). The expression in brain, stomach and gut was detected in embryos at E14.5. Of note, neither the liver nor the pancreas expressed OC-3 at E14.5 (Fig. 4B). The data in Table 2 summarize the patterns of expression of HNF-6, OC-2 and OC-3 mRNA in adult mouse organs. Except for brain and testis, all the organs that express ONECUT transcription factors are derived from the foregut and midgut endoderm. This suggests that OC-3 and OC-2 are involved in the control of endoderm development like HNF-6. Interestingly, expression of OC-3 and HNF-6 is mutually exclusive in endoderm-derived organs. Also, the stomach, gut and liver coexpress two members of the trio. Our finding that the properties of OC-3, OC-2 and HNF-6 are not completely superimposable suggests that they exert different regulatory roles in gene networks. However, in view of the similar DNA-binding specificity and partially overlapping expression pattern of these factors, their role is likely to be partially redundant.

Finally, our failure to find in the human genome other sequences similar to the single-copy *HNF6*, *OC2*, and *OC3* genes, makes it unlikely that more than three ONECUT transcription factors do exist in mammals.

## ACKNOWLEDGMENTS

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## REFERENCES

1. Lemaigre, F. P., Durvieux, S. M., Truong, O., Lannoy, V. J., Hsuan, J. J., and Rousseau, G. G. (1996) Hepatocyte nuclear factor 6, a transcription factor that contains a novel of homeodomain and a single cut domain. *Proc. Natl. Acad. Sci. USA* **93**, 9460–9464.
2. Lannoy, V. J., Bürglin, T. R., Rousseau, G. G., and Lemaigre, F. P. (1998) Isoforms of hepatocyte nuclear factor-6 differ in DNA-binding properties, contain a bifunctional homeodomain, and define the new ONECUT class of homeodomain proteins. *J. Biol. Chem.* **273**, 13552–13562.
3. Lannoy, V. J., Rodolosse, A., Pierreux, C. E., Rousseau, G. G., and Lemaigre, F. P. (2000) Transcriptional stimulation by hepatocyte nuclear factor-6. Target-specific recruitment of either CREB-binding protein (CBP) or p300/CBP-associated factor (pCAF). *J. Biol. Chem.* **275**, 22098–22103.
4. Rausa, F., Samadani, U., Honggang, Y., Lim, L., Fletcher, C. F., Jenkins, N. A., Copeland, N. G., and Costa, R. H. (1997) The cut-homeodomain transcriptional activator HNF-6 is coexpressed with its target gene HNF-3 $\beta$  in the developing murine liver and pancreas. *Dev. Biol.* **192**, 228–246.
5. Jacquemin, P., Lannoy, V. J., Rousseau, G. G., and Lemaigre, F. P. (1999) OC-2, a novel mammalian member of the ONECUT class of homeodomain transcription factors whose function in liver partially overlaps with that of hepatocyte nuclear factor-6. *J. Biol. Chem.* **274**, 2665–2671.
6. Møller, A. M., Ek, J., Durvieux, S. M., Urhammer, S. A., Clausen, J. O., Eiberg, H., Hansen, T., Rousseau, G. G., Lemaigre, F. P., and Pedersen, O. (1999) Hepatocyte nuclear factor-6: Associations between genetic variability and type II diabetes and between genetic variability and estimates of insulin secretion. *Diabetologia* **42**, 1011–1016.
7. Sasakura, Y., and Makabe, K. W. (2001) A gene encoding a new ONECUT class homeodomain protein in the ascidian *Halocynthia roretzi* functions in the differentiation and specification of neural cells in ascidian embryogenesis. *Mech. Dev.* **104**, 37–48.
8. Nguyen, D. N. T., Rohrbaugh, M., and Lai, Z.-C. (2000) The *Drosophila* homolog of Onecut homeodomain proteins is a neural-specific transcriptional activator with a potential role in regulating neural differentiation. *Mech. Dev.* **97**, 57–72.
9. Hong, S.-K., Kim, C.-H., Yoo, K.-W., Kim, H.-S., Kudoh, T., Dawid, I. B., and Huh, T.-L. (2002) Isolation and expression of a novel neuron-specific onecut homeobox gene in zebrafish. *Mech. Dev.* **112**, 199–202.
10. Landry, C., Clotman, F., Hioki, T., Oda, H., Picard, J. J., Lemaigre, F. P., and Rousseau, G. G. (1997) HNF-6 is expressed in endoderm derivatives and nervous system of the mouse embryo and participates to the cross-regulatory network of liver-enriched transcription factors. *Dev. Biol.* **192**, 247–257.
11. Jacquemin, P., Durvieux, S. M., Jensen, J., Godfraind, C., Gradwohl, G., Guillemot, F., Madsen, O. D., Carmeliet, P., Dewerchin,

- M., Collen, D., Rousseau, G. G., and Lemaigre, F. F. (2000) Transcription factor hepatocyte nuclear factor 6 regulates pancreatic endocrine cell differentiation and controls expression of the proendocrine gene *ngn3*. *Mol. Cell. Biol.* 20, 4445-4454.
12. Clotman, F., Lannoy, V. J., Reber, M., Cereghini, S., Cassiman, D., Jacquemin, P., Roskams, T., Rousseau, G. G., and Lemaigre, F. P. (2002) The onecut transcription factor HNF-6 is required for normal development of the biliary tract. *Development*, in press.
13. Jacquemin, P., Lannoy, V. J., O'Sullivan, J., Read, A., Lemaigre, F. P., and Rousseau, G. G. (2001) The transcription factor Onecut-2 controls the *microphthalmia*-associated transcription factor gene. *Biochem. Biophys. Res. Commun.* 280, 1200-1205.
14. Xiao, J. H., Davidson, I., Matthes, H., Garnier, J. M., and Chambon, P. (1991) Cloning, expression, and transcriptional properties of the human enhancer factor TEF-1. *Cell* 65, 551-568.
15. Rastegar, M., Szpirer, C., Rousseau, G. G., and Lemaigre, F. P. (1998) Hepatocyte nuclear factor 6: Organisation and chromosomal assignment of the rat gene and characterization of its promoter. *Biochem. J.* 334, 565-569.

## ATTACHMENT B

## The Transcription Factor Onecut-2 Controls the *Microphthalmia*-Associated Transcription Factor Gene

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*Microphthalmia*-associated transcription factor (*MITF*) is essential for melanocyte differentiation. *MITF* mutations are associated with some cases of Waardenburg syndrome (WS) type 2. WS is a dominantly inherited disease characterized by auditory-pigmentary defects that result from the absence of melanocytes. The lack of mutation in *MITF* coding sequences in some WS2 patients suggests that unidentified factors controlling *MITF* expression might be involved. We show here that the cut-homeodomain transcription factor Onecut-2 (OC-2) is expressed in melanocytes and binds to the *MITF* gene promoter. Overexpression of OC-2 in transfected cells stimulates *MITF* promoter activity. Mutations that prevent OC-2 binding decrease *MITF* promoter activity by 75%. Based on these results, we searched in 56 WS2 patients for mutations in the *OC2* gene or in OC-2 binding sites in the *MITF* promoter, but none was found. These results show that OC-2 stimulates *MITF* expression and that *OC2* is a candidate gene, but not a common cause, of WS. © 2001 Academic Press

**Key Words:** *microphthalmia*-associated transcription factor; Onecut; Waardenburg syndrome; melanocyte.

*Microphthalmia*-associated transcription factor (*MITF*) is essential for the commitment of the melanocyte lineage (1). Indeed, mice with mutations in *mitf* lack melanocytes (2, 3) and expression of *mitf* in fibroblasts converts them to melanocytes (4). In humans, defective melanocyte development causes the Waardenburg syndrome (WS). This is a dominantly-inherited disease with congenital sensorineural hearing loss and pigmentation defects (reviewed in Refs. 5, 6). Four subtypes of WS have been described. WS type 2 (WS2) is associated with mutations of *MITF* (7, 8). Other sub-

types are associated with mutations of *PAX3* (WS1 and 3; Refs. 9, 10), of *SOX10* (WS4; Ref. 11), or of endothelin B or endothelin-B receptor (WS4; Refs. 12–14). Since Pax-3 and Sox-10 regulate *MITF* promoter activity, it has been suggested that mutations of these two factors cause the pigmentary defects observed in WS3 and WS4 via a secondary deficiency in *MITF* (15–20). Only a small proportion of the WS2 patients have mutations in the *MITF* coding sequence (5). Thus, unexplained cases of WS2 could result from mutations in unknown transcription factors that control the *MITF* gene or in binding sites for such factors in the *MITF* promoter. The identification of such factors would provide novel candidate genes for WS. In this context, transcription factors that are expressed in skin are of obvious interest. We have cloned one such factor which we called Onecut-2 (OC-2, Ref. 21).

OC-2 belongs to the ONECUT class of homeodomain transcription factors characterized by a bipartite DNA-binding region composed of a single cut domain and a divergent homeodomain (22). In this work we have identified OC-2 as a novel transcriptional stimulator of *MITF* expression in melanocytes. We have also explored the possibility that mutations in the *OC2* gene or in OC-2 binding sites in the *MITF* gene promoter are associated with WS2.

### MATERIALS AND METHODS

**Reverse-transcription PCR (RT-PCR).** To detect OC-2 mRNA in melanocytes and melanoma cell lines, 1 µg of total RNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase and random hexamers (Life Technologies, Inc.). Human OC-2 cDNA was amplified by PCR and the specificity of the amplified products was verified in Southern blotting, as described (21, 23). The integrity of the RNA preparations was controlled by amplification of a  $\beta$ -actin cDNA fragment. Negative controls included RT-PCR performed without reverse transcriptase.

**Expression vector and reporter constructs.** The expression vector pXJ42-OC2 has been described (21). For the reporter constructs, the *MITF* promoter regions were cloned into the luciferase reporter

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vector pGL3-Basic (Promega). The proximal region of the *MITF* promoter, extending from +95 to -382 (24), was obtained by PCR using specific primers with *HindIII* and *NheI* restriction sites at their extremity. A genomic fragment of the human *MITF* gene cloned in pBluescript (24) was used as template. The amplified fragment was cloned in the *HindIII* and *NheI* sites of pGL3-Basic to produce pMITF-P-luc and sequenced to check for the absence of PCR-generated mutations. The region of the *MITF* promoter from -2008 to -2259 was obtained by PCR using specific primers with *NheI* and *SmaI* restriction sites at their extremity and human genomic DNA as template. The 252-bp amplified fragment was inserted in the *EcoRV* site of pBluescript by TA cloning. The resulting plasmid was digested by *NheI* and *KpnI* (site present in the pBluescript polylinker) to produce the distal *MITF* fragment which was cloned into the corresponding sites of pMITF-P-luc to produce pMITF-P/D-luc. Mutations in the *MITF* fragments were generated by site-directed mutagenesis according to a PCR strategy described earlier (25). The mutations were identical to those of the mutated oligonucleotides used in electrophoretic mobility shift assays (EMSA) (see Fig. 2). Mutated fragments were cloned in the reporter plasmids to produce pMITF-Pmut-luc, pMITF-P/Dmut-luc, pMITF-Pmut/D-luc and pMITF-Pmut/Dmut-luc. All constructs were sequenced. The internal control vector pRL-138 (22) contains the *p/k2* gene promoter (-138 to +86) cloned in pRLnull upstream of the Renilla luciferase coding sequence (Promega).

**Cells.** Normal human epidermal melanocytes, derived from fore-skin, were obtained from PromoCell. The 397-mel and 526-mel cell lines were established at the National Cancer Institute (26), and the LB-373-MEL, BB74-MEL, and LB1622-MEL cell lines at the Ludwig Institute of Cancer Research (Brussels, Belgium) (27).

**Transfections and cell extracts.** Human melanoma 397-mel and cervix carcinoma HeLa cell lines were grown in Iscove's modified Dulbecco's medium and in Dulbecco's modified Eagle medium (DMEM), respectively. Both media were supplemented with 10% fetal calf serum (FCS). Cells ( $1 \times 10^5$  cells per well on 24-well plates) were transfected in medium without FCS by lipofection using Lipofectamine-PLUS (Life Technologies, Inc.), 400 ng of the corresponding reporter vector, 15 ng of the expression vector pXJ42-OC2 and 15 ng of pRL-138 as internal control. After 4 h, the cells were washed with phosphate-buffered saline (PBS) and further incubated for 45 h in the corresponding medium plus 10% FCS before measuring luciferase activities with the dual-luciferase kit (Promega). Luciferase activities were measured with a TD-20/20 luminometer (Promega) and expressed as the ratio of reporter activity (firefly luciferase) to internal control activity (*Renilla* luciferase).

COS-7 cells ( $3 \times 10^5$  cells per 6-cm dish) were transfected in DMEM without FCS by lipofection using *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-triethylammonium methylsulfate (DOTAP, Roche Molecular Biochemicals) and 5  $\mu$ g of pXJ42-OC2. Forty-eight hours after transfection, the cells were washed with PBS and harvested in 1 ml of 40 mM Tris-Cl (pH 7.5), 1 mM EDTA, 150 mM NaCl. The cells were pelleted and resuspended in 60  $\mu$ l of 50 mM Tris-Cl (pH 7.9), 500 mM KCl, 0.5 mM EDTA, 2.5 mg/ml leupeptin, 1 mM dithiothreitol, 0.1% (v/v) Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 20% (v/v) glycerol. After three freeze-thaw cycles, the lysates were centrifuged and the supernatants collected.

**Electrophoretic mobility shift assays (EMSA).** COS-7 cells lysates (5  $\mu$ l) were incubated on ice for 20 min in a final volume of 20  $\mu$ l containing 10 mM Hepes (pH 7.6), 1 mM dithiothreitol, 1 mM MgCl<sub>2</sub>, 0.5 mM ECTA, 60 mM KCl, 10% (v/v) glycerol, 4  $\mu$ g of poly(dI-dC), and the <sup>32</sup>P-labeled probe (30,000 cpm). The samples were loaded on a 7% acrylamide gel (acrylamide/bisacrylamide ratio was 29:1) in 0.25 $\times$  TBE buffer and electrophoresed at 200 V.

**Search for mutations in type 2 Waardenburg patients.** The coding sequence of the human *OC2* gene was amplified in 10 overlapping fragments with the following primer pairs: HFM2.13, 5'-CCTGATGGACTGAATGAAGG-3' and HFM2.9, 5'-TGGGGCTGGC-

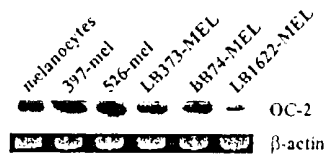


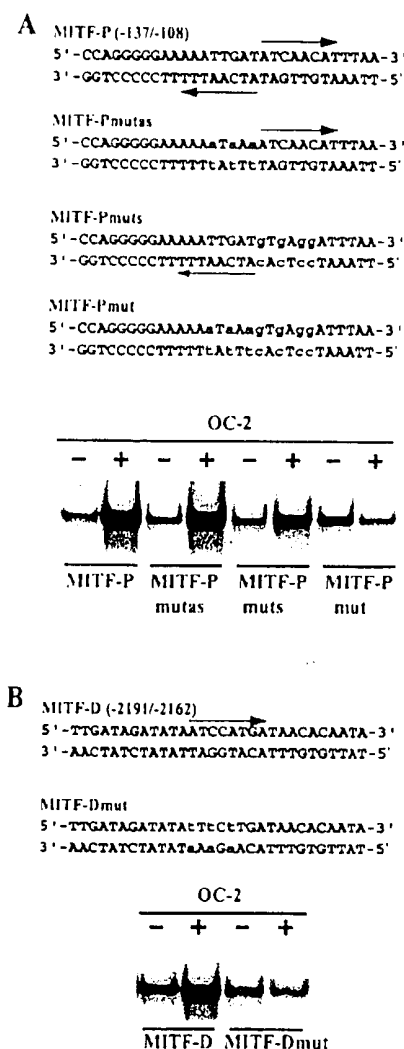
FIG. 1. OC2 is expressed in human melanocytes and melanoma cell lines. The amount of OC2 mRNA was determined by RT-PCR and the specificity of the amplified products was controlled by Southern blotting.  $\beta$ -Actin mRNA was similarly amplified as a control for RNA integrity. The products were visualized on a nondenaturing acrylamide gel stained with ethidium bromide.

CAGCAGCT-3'; HFM2.26, 5'-GGGCCATGAGCAGGAGC-3' and HFM2.25, 5'-GTGCAGCGGATGGAGAG-3'; HFM2.14, 5'-GCCATGGTACCAGCAT-3' and HFM2.23, 5'-AGGGTGGTGAAGTGT-CAG-3'; HFM2.24, 5'-TGAGCAACACCTACACCAC-3' and HFM2.7, 5'-GTAGAGGTTGTTCATGGCC-3'; HFM2.15, 5'-CACCACGCGCCTGTCCGGC-3' and HFM2.19, 5'-CGTCCGAAGTTGGGGCTGAG-3'; HFM2.18, 5'-GTCTGCCCAACTACGGTCC-3' and HFM2.16, 5'-GGCACTGGGTGCCAGCAC-3'; HFM2.20, 5'-ACACTCAGTCTCACGGC-3' and HFM2.3, 5'-GTTTACTCCACGGTTTGG-3'; HFM2.21, 5'-GGGTGCTGTGCCGGTCTC-3' and E11hOC2, 5'-GAACCCGGTGTTCGTGGC-3'; H2hOC2, 5'-TTTCCATGGATCACCTTCTC-3' and HFM2.22, 5'-GTTGCTGACGGTTGTGAG-3'; HFM2.1, 5'-ATTTCCAGCAGCTGGG-3' and HFM2.2A, 5'-CAGCTAGGAATCCGGTCTC-3'. The promoter of the human *MITF* gene was amplified in two overlapping fragments using primers MITF-ProxR (5'-AAAGATGATAGTGAATTGGCC-3'), MITF-ProxR (5'-ATTCCAATTGAAGATGGACTG-3'), MITF-DisR (5'-TCCTGATGTGAGGTCAATAGGC-3') and MITF-DisR (5'-GTCAGATCAAGGCCAATTCACT-3'). The MITF proximal PCR product (303 bp) comprises nucleotides -232 to +70. This fragment contains one Pax-3 and two OC-2 binding sites. The MITF distal product (320 bp) comprises nucleotides -530 to -211. This fragment contains a putative Pax-3 binding site and a Sox-10 binding site. PCR products were analyzed by SSCP-heteroduplex analysis in silver-stained polyacrylamide gels as described (5). Any samples giving abnormal bands were sequenced in both directions on an ABI 373 sequencer.

## RESULTS AND DISCUSSION

*OC-2 is expressed in melanocytes and binds to the human MITF promoter.* As OC2 is expressed in human skin (21) we determined whether it could be detected in melanocytes or melanoma cell lines. As shown in Fig. 1, this was the case. A computer search using the BLAST algorithm to find OC-2 targets identified putative binding sites in the human *MITF* gene. The *MITF* promoter contains sequences that match the 8-bp (5'-A/G-T-C-A/C/G-A-T-N-A/T/G-3') consensus for Onecut factor binding (22 and our personal observations). In the proximal region (MITF-P), two potential binding sites were found, one on the sense strand (-119/-112), one on the antisense strand (-120/-127) (Fig. 2A). In the distal promoter region (MITF-D), a potential site was also found (-2179/-2172) (Fig. 2B).

Binding of OC-2 was tested by EMSA with extracts from COS-7 cells transfected with an OC-2 expression vector. Extracts from nontransfected COS-7 cells, which are devoid of OC-2, served as control. Figure 2A



**FIG. 2.** OC-2 binds to the *MITF* gene promoter. Sequence of the proximal (A) and distal (B) OC-2 binding sites in the *MITF* promoter. The Onecut binding consensus is delineated by arrows and the mutated nucleotides in the probes MITF-Pmutas, MITF-Pmut, MITF-Pmut and MITF-Dmut are indicated in lowercase letters. EMSA were performed with extracts from nontransfected COS-7 cells or from COS-7 cells transfected with the OC-2 expression vector, as indicated above the lanes. The radioactive probes used are indicated below the lanes.

shows that OC-2 bound to the proximal sites (MITF-P). Control extracts produced a nonspecific complex that migrated similarly to the OC-2/DNA complex. To determine the relative contribution of the two proximal sites, we mutated the corresponding sequences. After destroying the site on the antisense strand alone (MITF-Pmutas), binding of OC-2 persisted (Fig. 2A). This showed that the site on the sense strand can bind

OC-2. Destruction of the latter site alone (MITF-Pmut) marginally affected OC-2 binding (Fig. 2A), indicating that the site on the antisense strand also binds OC-2. Destruction of both sites (MITF-Pmut) was required to abolish binding of OC-2 (Fig. 2A). These results, and the absence of trimeric complexes, showed that in the intact promoter a single OC-2 molecule binds to either one of the two proximal sites. We tested similarly whether OC-2 binds to the distal Onecut binding sequence in the *MITF* promoter (MITF-D). This was the case (Fig. 2B). Destruction of this site by mutagenesis abolished binding (Fig. 2B). We concluded that OC-2 binds to two regions, separated by about 2 kb, in the *MITF* promoter.

The proximal OC-2 binding region is important for the activity of the *MITF* promoter. To test the role of the OC-2 sites in *MITF* promoter activity, the promoter regions containing these sites were cloned upstream of the luciferase reporter gene. pMITF-P-luc contains the *MITF* promoter region from +95 to -382 and encompasses the two proximal OC-2 sites. pMITF-P/D-luc contains in addition the -2008 to -2259 *MITF* promoter region that encompasses the distal site (Fig. 3). These constructs were transfected into 397-mel cells, which express OC-2 as demonstrated in Fig. 1. The pMITF-P-luc construct showed a strong promoter activity in 397-mel cells, more than 100-fold higher than that of pGL3-Basic (Fig. 3). A similar activity was found for pMITF-P/D-luc, showing that the distal region does not contribute to the activity of the promoter under our experimental conditions. When the proximal OC-2 binding sites were destroyed (pMITF-Pmut-luc, same mutations as in probe MITF-Pmut), there was a 4-fold decrease in activity, suggesting that OC-2 binding to these sites contributes 75% of the activity of the promoter in these melanoma cells (Fig. 3). Mutation of the distal OC-2 site alone (pMITF-P/Dmut-luc, same mutations as in probe MITF-Dmut) did not affect promoter activity, in keeping with the lack of influence described above of OC-2 binding to the distal region of the *MITF* gene. Consistent with this, destroying the distal site did not reduce the activity of the construct mutated in the proximal sites (pMITF-Pmut/Dmut-luc, Fig. 3). We concluded that the integrity of the proximal OC-2 binding sites is crucial for full activity of the *MITF* promoter.

OC-2 can stimulate the *MITF* promoter. To confirm that OC-2 can stimulate the *MITF* promoter, we co-transfected the pMITF-P-luc reporter construct and the OC-2 expression vector in HeLa cells (Fig. 4). Expression of OC-2 increased the activity of pMITF-P-luc when compared with the activity of this construct co-transfected with the empty expression vector. No stimulation of the pMITF-Pmut-luc construct by OC-2 was observed, indicating that the activity of OC-2 on the



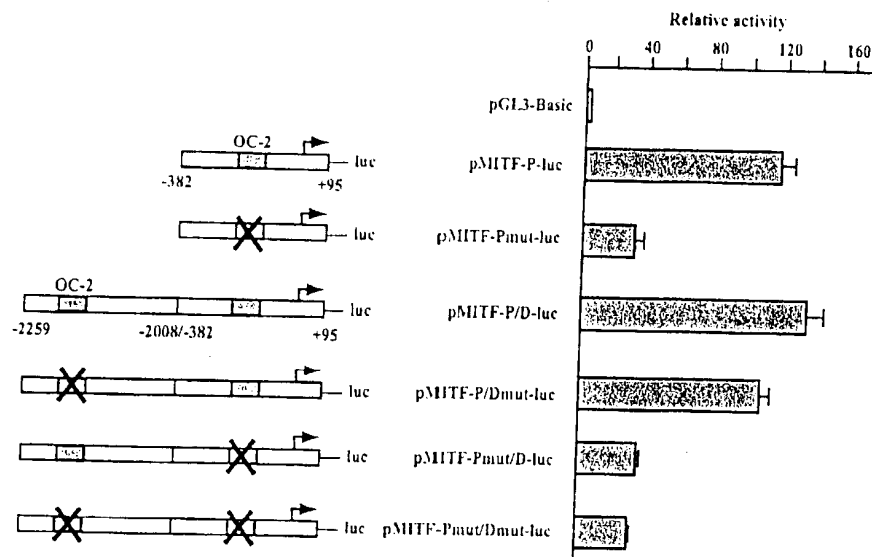


FIG. 3. The proximal OC-2 binding sites control *MITF* promoter activity. Melanoma (397-mel) cells were transiently transfected with the *MITF* promoter-firefly luciferase reporter constructs indicated or with the empty vector (pGL3-basic) as a control. Mutations in the OC-2 binding sites (crosses) were as described in the legend to Fig. 2. Firefly luciferase values were normalized for *Renilla* luciferase values from the internal control plasmid pRL-138. A relative activity of 1 was assigned to pGL3 Basic (means  $\pm$  SE,  $n = 3$ ).

*MITF* promoter is mediated by the proximal binding sites.

*Search for mutations in OC2 or in OC-2 binding sites as a potential cause for WS2.* A subset of WS2 is caused by haploinsufficiency for *MITF* (5, 8). Moreover, those WS2 patients do not appear clinically distinct from those without *MITF* mutation. Our demonstration that OC-2 controls transcription of the *MITF* gene suggested that loss-of-function mutations in *OC2* might be one cause of WS2. To check this we tested DNA from a panel of 56 unrelated patients who fitted

current diagnostic criteria for WS2 (28). These had been referred for *PAX3* and *MITF* mutation testing because of a possible diagnosis of Waardenburg syndrome, but no *PAX3* or *MITF* mutation had been detected. They included a variety of phenotypes, but all had hearing loss and either pigmentary anomalies (typically white forelock, early graying or heterochromia irides) or a family history of pigmentary anomaly. The genomic *OC2* coding sequence was amplified in 10 overlapping amplicons. PCR products were examined by combined SSCP-heteroduplex analysis on silver-stained gels and any product giving abnormal bands was sequenced. Nine samples gave a variant band in amplicon 4. Sequencing revealed the amplicon 4 variant as a silent change (1098T>C, F337F). Individuals with this variant, in heterozygous form in each case, came from a diversity of genetic backgrounds. This polymorphism was also found in 16/200 chromosomes of healthy controls. No other *OC2* variant was detected in our panel.

We also investigated by SSCP-heteroduplex analysis of PCR products whether WS2 might be caused by mutations in the functional OC2 binding sites of the *MITF* promoter. We amplified a 303-bp fragment from the proximal part of the *MITF* promoter (positions -232 to +70) which contains the two functional OC-2 binding sites studied here and a *PAX3* site at -40 to -26 (16). Additionally we amplified a 320-bp distal fragment (-530 to -211) that includes a putative

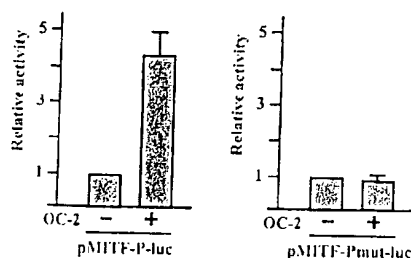


FIG. 4. OC-2 stimulates transcription from the *MITF* gene promoter. HeLa cells were transiently transfected with the OC-2 expression vector or with the empty vector (pCMV-NH) as a control. The cotransfected firefly luciferase reporter constructs pMITF-P-luc and pMITF-Pmut-luc were used as indicated. Firefly luciferase values were normalized for *Renilla* luciferase values from the internal control plasmid pRL-138. A relative activity of 1 was assigned to the empty vector (means  $\pm$  SE,  $n = 3$ ).

Pax-3 binding site at -260 to -244 (15) and a Sox-10 site at -268 to -262 (16). DNA samples from the same 56 WS2 patients were tested, but no mutations in the *MITF* promoter were found.

In conclusion, our data suggest that mutations in OC2 or in OC-2 binding sites in the *MITF* promoter may occasionally cause an auditory-pigmentary syndrome, but they exclude such mutations as a frequent cause of WS2. Our data also demonstrate that OC-2 is essential for *MITF* promoter activity. Indeed, OC-2 stimulated the *MITF* promoter and mutations in the OC-2 binding sites led to a 75% decrease in *MITF* promoter activity. Work by others has shown that the loss of Pax-3 (15), Sox-10 (20), LEF-1 (29) or CREB (30) binding to the *MITF* promoter also severely affected its activity. This indicates that integration of the control exerted by each individual transcription factor is essential to obtain adequate *MITF* promoter activity. Finally, since Pax-3 and Sox-10 control melanocyte differentiation (31), at least in part via their effect on *MITF* expression, we suggest that OC-2 is also involved in the control of melanocyte differentiation.

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#### REFERENCES

- Opdecamp, K., Nakayama, A., Nguyen, M. T., Hodgkinson, C. A., Pavan, W. J., and Arnheiter, H. (1997) Melanocyte development *in vivo* and in neural crest cell cultures: Crucial dependence on the Mitf basic-helix-loop-helix zipper transcription factor. *Development* 124, 2377-2386.
- Steingrimsson, E., Moore, K. J., Lamoreux, M. L., Ferre-D'Amare, A. R., Burley, S. K., Zimring, D. C., Skow, L. C., Hodgkinson, C. A., Arnheiter, H., Copeland, N. G., et al. (1994) Molecular basis of mouse microphthalmia (mi) mutations helps explain their developmental and phenotypic consequences. *Nat. Genet.* 8, 256-263.
- Moore, K. J. (1995) Insight into the microphthalmia gene. *Trends Genet.* 11, 442-448.
- Tachibana, M., Takeda, K., Nobukuni, Y., Urabe, K., Long, J. E., Meyers, K. A., Aaronson, S. A., and Miki, T. (1996) Ectopic expression of MITF, a gene for Waardenburg syndrome type 2, converts fibroblasts to cells with melanocyte characteristics. *Nat. Genet.* 14, 50-54.
- Tassabehji, M., Newton, V. E., Liu, X. Z., Brady, A., Donnai, D., Krajewska-Walasek, M., Murday, V., Norman, A., Obersztyn, E., Reardon, W., et al. (1995) The mutational spectrum in Waardenburg syndrome. *Hum. Mol. Genet.* 4, 2131-2137.
- Read, A. P., and Newton, V. E. (1997) Waardenburg syndrome. *J. Med. Genet.* 34, 656-665.
- Tassabehji, M., Newton, V. E., and Read, A. P. (1994) Waardenburg syndrome type 2 caused by mutations in the human microphthalmia (*MITF*) gene. *Nat. Genet.* 8, 251-255.
- Nobukuni, Y., Watanabe, A., Takeda, K., Skarka, H., and Tachibana, M. (1996) Analyses of loss-of-function mutations of the *MITF* gene suggest that haploinsufficiency is a cause of Waardenburg syndrome type 2A. *Am. J. Hum. Genet.* 59, 76-83.
- Tassabehji, M., Read, A. P., Newton, V. E., Patton, M., Gruss, P., Harris, R., and Strachan, T. (1993) Mutations in the *PAX3* gene causing Waardenburg syndrome type 1 and type 2. *Nat. Genet.* 3, 26-30.
- Hoth, C. F., Milunsky, A., Lipsky, N., Sheffer, R., Clarren, S. K., and Baldwin, C. T. (1993) Mutations in the paired domain of the human *PAX3* gene cause Klein-Waardenburg syndrome (WS-III) as well as Waardenburg syndrome type I (WS-I). *Am. J. Hum. Genet.* 52, 455-462.
- Pingault, V., Bondurand, N., Kuhlbrodt, K., Goerich, D. E., Prehu, M. O., Puliti, A., Herbarth, B., Hermans-Borgmeyer, I., Legius, E., Matthijs, G., Amiel, J., Lyonnet, S., Ceccherini, I., Romeo, G., Smith, J. C., Read, A. P., Wegner, M., and Goossens, M. (1998) *SOX10* mutations in patients with Waardenburg-Hirschsprung disease. *Nat. Genet.* 18, 171-173.
- Attie, T., Till, M., Pelet, A., Amiel, J., Edery, P., Boutrand, L., Munnich, A., and Lyonnet, S. (1995) Mutation of the endothelin-receptor B gene in Waardenburg-Hirschsprung disease. *Hum. Mol. Genet.* 4, 2407-2409.
- Edery, P., Attie, T., Amiel, J., Pelet, A., Eng, C., Hofstra, R. M., Martelli, H., Bidaud, C., Munnich, A., and Lyonnet, S. (1996) Mutation of the endothelin-3 gene in the Waardenburg-Hirschsprung disease (Shah-Waardenburg syndrome). *Nat. Genet.* 12, 442-444.
- Hofstra, R. M., Osinga, J., Tan-Sindhunata, G., Wu, Y., Kamsteeg, E. J., Stulp, R. P., van Ravenswaaij-Arts, C., Majoor-Krakauer, D., Angrist, M., Chakravarti, A., Meijers, C., and Buys, C. H. (1996) A homozygous mutation in the endothelin-3 gene associated with a combined Waardenburg type 2 and Hirschsprung phenotype (Shah-Waardenburg syndrome). *Nat. Genet.* 12, 445-447.
- Watanabe, A., Takeda, K., Ploplis, B., and Tachibana, M. (1998) Epistatic relationship between Waardenburg syndrome genes *MITF* and *PAX3*. *Nat. Genet.* 18, 283-286.
- Bondurand, N., Pingault, V., Goerich, D. E., Lemort, N., Sock, E., Caignec, C. L., Wegner, M., and Goossens, M. (2000) Interaction among *SOX10*, *PAX3* and *MITF*, three genes altered in Waardenburg syndrome. *Hum. Mol. Genet.* 9, 1907-1917.
- Goding, C. R. (2000) Mitf from neural crest to melanoma: Signal transduction and transcription in the melanocyte lineage. *Genes Dev.* 14, 1712-1728.
- Lee, M., Goodall, J., Verastegui, C., Ballotti, R., and Goding, C. R. (2000) Direct regulation of the Microphthalmia promoter by Sox10 links Waardenburg-Shah syndrome (WS4)-associated hypopigmentation and deafness to WS2. *J. Biol. Chem.* 275, 37978-37983.
- Potterf, S. B., Furumura, M., Dunn, K. J., Arnheiter, H., and Pavan, W. J. (2000) Transcription factor hierarchy in Waardenburg syndrome: Regulation of MITF expression by *SOX10* and *PAX3*. *Hum. Genet.* 107, 1-6.
- Verastegui, C., Bille, K., Ortonne, J. P., and Ballotti, R. (2000) Regulation of the microphthalmia-associated transcription factor gene by the Waardenburg syndrome type 4 gene, *SOX10*. *J. Biol. Chem.* 275, 30757-30760.
- Jacquemin, P., Lannoy, V. J., Rousseau, G. G., and Lemaigre, F. P. (1999) OC-2, a novel mammalian member of the ONECUT

- class of homeodomain transcription factors whose function in liver partially overlaps with that of hepatocyte nuclear factor-6. *J. Biol. Chem.* **274**, 2665-2671.
22. Lannoy, V. J., Burglin, T. R., Rousseau, G. G., and Lemaigre, F. P. (1998) Isoforms of hepatocyte nuclear factor-6 differ in DNA-binding properties, contain a bifunctional homeodomain, and define the new ONECUT class of homeodomain proteins. *J. Biol. Chem.* **273**, 13552-13562.
23. Jacquemin, P., Martial, J. A., and Davidson, I. (1997) Human TEF-5 is preferentially expressed in placenta and binds to multiple functional elements of the human chorionic somatomammotropin-B gene enhancer. *J. Biol. Chem.* **272**, 12928-12937.
24. Fuse, N., Yasumoto, K., Suzuki, H., Takahashi, K., and Shibahara, S. (1996) Identification of a melanocyte-type promoter of the microphthalmia-associated transcription factor gene. *Biochem Biophys Res Commun.* **219**, 702-707.
25. Jacquemin, P., Hwang, J. J., Martial, J. A., Dolle, P., and Davidson, I. (1996) A novel family of developmentally regulated mammalian transcription factors containing the TEA/ATTS DNA binding domain. *J. Biol. Chem.* **271**, 21775-21785.
26. Kang, X., Robbins, P. F., Fitzgerald, E. B., Wang, R., Rosenberg, S. A., and Kawakami, Y. (1997) Induction of melanoma reactive T cells by stimulator cells expressing melanoma epitope-major histocompatibility complex class I fusion proteins. *Cancer Res.* **57**, 202-205.
27. Brasseur, F. (1999) Melanoma: Brussels melanoma cell lines. In *Human Cell Culture* (Masters, J. R. W., and Pauson, B., Eds.), Vol. I, pp. 275-282, Kluwer Academic, Great Britain.
28. Liu, X. Z., Newton, V. E., and Read, A. P. (1995) Waardenburg syndrome type II: Phenotypic findings and diagnostic criteria. *Am. J. Med. Genet.* **55**, 95-100.
29. Takeda, K., Yasumoto, K., Takada, R., Takada, S., Watanabe, K., Udono, T., Saito, H., Takahashi, K., and Shibahara, S. (2000) Induction of melanocyte-specific microphthalmia-associated transcription factor by Wnt-3a. *J. Biol. Chem.* **275**, 14013-14016.
30. Bertolotto, C., Abbe, P., Hemesath, T. J., Bille, K., Fisher, D. E., Ortonne, J. P., and Ballot, R. (1996) Microphthalmia gene product as a signal transducer in cAMP-induced differentiation of melanocytes. *J. Cell Biol.* **142**, 827-835.
31. Hornyak, T. J., Hayes, D. J., Chiu, L., and Ziff, E. B. (2001) Transcription factors in melanocyte development: Distinct roles for Pax-3 and Mitf. *Mech. Dev.* **101**, 47-59.

## ATTACHMENT C

## CERTIFICATION OF TRANSLATION

"PHARMACEUTICAL COMPOSITION FOR THE TREATMENT OR PREVENTION OF  
DIABETES OR CANCER"

I, Jennifer KILBEE,

c/o Technisches Fachübersetzungsbüro, Försterweg 33, A-2136

Laa/Thaya, Austria,

am the translator of the documents attached and certify that  
the following is a true translation to the best of my knowledge  
and belief.

*Jennifer Kilbee*

Signature of translator dated this 15<sup>th</sup> day of September 2003



PHARMACEUTICAL COMPOSITION FOR THE TREATMENT OR PREVENTION OF  
DIABETES OR CANCER

OBJECT OF THE INVENTION

The present invention relates to a novel pharmaceutical composition for the treatment or prevention of diabetes or cancer, in particular a cellular therapy for diabetes through the creation of an artificial pancreas.

TECHNOLOGICAL BACKGROUND OF THE BASIS OF THE INVENTION

Diabetes is a generic term under which are designated disorders characterized by a combination of polyuria and polydipsia. Diabetes mellitus, hereinafter also called sugar diabetes, which may be of a type 1 or type 2, is due to a poor functioning of the beta cells of the endocrine pancreas (islets of Langerhans) which synthesizes and secretes insulin (Gerich & Haefliger, COED 5, pp. 144-148 (1998)). It is often accompanied (type 2 diabetes) by a resistance of the shock tissues to the action of insulin.

Sugar diabetes is one of the most common metabolic disorders, particularly in the industrialized world (Leahy, COED 5, pp. 73-74 (1998)). It is characterized by a deficiency in the utilization of glucose and may have serious and sometimes fatal pathological consequences, such as metabolic, cardiovascular and neurological problems, and lesions to the retina or kidneys. Treatment by insulin requires one or more daily injections for life.

Consequently, a definite need exists for replacing these injections with transplantable systems (Gage et al., *Nature* 392, Supplement 3 (1998)).

PRIOR ART

The document Lemaigre et al. (1996) describes a cDNA encoding the hepatocyte nuclear factor-6, hereinafter called HNF-6. This protein controls the transcription of certain genes in a small number of tissues where it is expressed (Samadani & Costa (1996)). The expression of this molecule was particularly identified in the pancreas of mice (Landry et al. (1997) and Rausa et al. (1997)).

The HNF-6 protein contains two domains for binding to DNA, one domain called cut and one domain called homeo, characteristic of HNF-6 by the presence of a phenylalanine in position 48 and a methionine in position 50 (hereinafter called F48M50 dyad).

French Patent Application FR-2,696,755 describes an implantable capsule comprising an external envelope consisting of a hydrogel of acrylonitrile and sodium methallylsulfonate, an internal center comprising an encapsulated substance, which may consist of islets of Langerhans, beta pancreatic cells, or hepatocytes. The envelope is a biocompatible membrane selectively permeable to insulin or to the nutrients necessary to the encapsulated substance. This product may be utilized in the transplantation of cells or groups of cells such as islets of Langerhans for mitigating the insufficient production of insulin in diabetic patients.

International Patent Application WO95/09231 describes novel insulin-secreting beta cell lines which may appear in the form of "pseudoislets" and may be encapsulated in a biocompatible hydrogel; and possibly incorporated in the transplantable fibers intended for subcutaneous or intraperitoneal introduction in the patient for treating insulin-dependent patients.

International Patent Application WO95/29988 describes a procedure for culturing cell lines; particularly pancreatic cells, likely to create in vivo reimplantable cell islets in mammals so as to treat pancreatic illnesses in humans or animals.

#### GOALS OF THE INVENTION

The present invention aims to provide a novel pharmaceutical composition designed to be utilized in the prevention or treatment of diabetes or cancer and may be utilized either in the field of genetic therapy, or in the field of cellular therapy, by producing cellular masses or forming an artificial pancreatic tissue or organ.

### CHARACTERISTIC ELEMENTS OF THE INVENTION

The inventors unexpectedly discovered that the invalidation of the HNF-6 gene in mice shows that this gene is essential for the function of islets of Langerhans and for the insulin response of the organism. Furthermore, the inventors have shown that other proteins similar to HNF-6, which share two of HNF-6's special features, firstly the presence of a single cut domain and secondly the presence of the F48M50 dyad in the homeo domain (Lannoy et al. (1998)) belong to the same ONECUT family (summarized in OC) (Lannoy et al. (1998)), also involved in certain essential metabolic mechanisms. Among the protein family thus defined, which includes the HNF-6 protein and the OC-2 protein among others, certain proteins have essential functions in animals, particularly in humans, particularly in glucose metabolism. Furthermore, such molecules may be utilized in treating a certain number of conditions and diseases, particularly diabetes or cancer, and preferably melanoma.

The present invention thus relates to a pharmaceutical composition comprising an appropriate pharmaceutical vehicle and an element chosen from the group consisting of a nucleotide sequence encoding a ONECUT family member protein, particularly HNF-6 or the OC-2 factor wherein the nucleotide and peptide sequence is hereinafter described (Figures 1a and 1b), a vector comprising the said nucleotide sequence, the encoded polypeptide sequence and/or a cell line transformed by the said vector and expressing these said nucleotide sequences, particularly likely to synthesize HNF-6 or another member of the ONECUT family such as the OC-2 factor.

"Nucleotide sequence encoding HNF-6" is understood to be the encoding sequence corresponding to the cDNA HNF-6 sequence such as already described, particularly by Lemaigre et al. (1996), and the equivalent human or animal sequences likely to hybridize with this cDNA. This hybridization is preferably carried out under stringent conditions so as to identify the different genomic sequences encoding a sequence of amino acids identical or similar to that of HNF-6 or OC-2. Particularly, these are other specific sequences from other mammals that have the same function, but are different particularly as regards redundancy of the genetic code. The standard hybridization conditions are preferably the following: hybridization at 40 °C in 50 % of formamide, 5 x SSC 20 mM sodium phosphate, pH 6.8, cleaning in 0.2 x SSC at 50 °C. Modifications to these conditions according to the length and content of GC nucleotides in the



sequence to be hybridized may be proposed by a person skilled in the art. Other hybridization conditions are, among others, those described by Sambrook et al., §§ 9.47-9.51 in *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, Laboratory Press, Cold Spring Harbor, New York (1989).

According to the invention, the gene encoding HNF-6 utilized concerns genomic sequences encoding the two alpha and beta isoforms of HNF-6 such as described by Lannoy et al. (1998).

The pharmaceutical composition of the invention may be utilized for obtaining a genetic and/or cellular therapy for a patient at risk for developing diabetes or suffering from diabetes, or at risk for developing cancer or suffering from cancer, particularly melanoma. In the genetic therapy field, the nucleotide sequence of the invention may be administrated to the patient or to the cell lines of the patient by a standard ex vivo treatment by procedures well known to a person skilled in the art or through a vector, preferably chosen from among the group consisting of plasmids, viruses, phagemids, and lipid vesicles such as cationic lipids, liposomes or a mixture thereof. The vector will incorporate all the elements necessary for obtaining the expression of the nucleotide sequence according to the invention in the patient, preferably in the specific cell lines to be treated, such as the pancreatic cells involved in the synthesis of insulin, hepatic cells involved in insulin response or epidermal cells or dermal cells at risk for developing melanoma.

The pharmaceutical composition of the invention may also be utilized in cellular therapy by directly injecting cells in an in vivo or ex vivo procedure or by forming an artificial cell mass such as described in Patent Applications FR-2,696,755, WO95/09231 and WO95/29988. It is possible to obtain proliferation of the cells transformed by the nucleotide sequence of the invention or by the vector of the invention by procedures well known to a person skilled in the art, in particular those described in Patent Applications WO97/49728 and WO95/29988.

The pharmaceutical vehicle according to the invention varies according to the delivery system chosen (intravenous, intramuscular, oral, etc.) and is an excipient well known to a person skilled in the art, appearing in the form of tablets, pills, capsules, solutions, syrups, etc. This component may possibly comprise adjuvants (particularly a growth

hormone) well known to a person skilled in the art so as to introduce synergistic effects or to suppress certain specific cellular or immune reactions so as to reduce certain undesirable side effects or toxic effects from the active ingredient or vehicle of the invention.

The percentage of the active ingredient (nucleotide sequence, amino acid sequence or fragments thereof, vector, cell line, etc.) in the pharmaceutical composition may vary according to a very wide range, uniquely limited by the frequency of administration, tolerance to and level of acceptance of the composition according to the invention by the patient.

The present invention also concerns the utilization of the pharmaceutical composition of the invention in preparing medications intended for the treatment and/or prevention of type 1 or type 2 diabetes, conditions associated with diabetes, particularly conditions associated with the poor function of endocrine pancreas beta cells which synthesize and secrete insulin, and/or for the treatment of cancer, particularly melanoma.

A final aspect of the present invention concerns the method of treating the patient, particularly a patient at risk for developing diabetes, suffering from diabetes or at risk for developing cancer or suffering from cancer, particularly melanoma, for which the pharmaceutical composition of the invention is administered to said patient by an in vivo or ex vivo method of treatment.

## REFERENCES

- Kahn, B.B., Type 2 Diabetes: when insulin secretion fails to compensate for insulin resistance. *Cell* 92, pp. 593-596 (1998)
- Landry, C. et al., HNF-6 is expressed in endoderm derivatives and nervous system of the mouse embryo and participates to the cross-regulatory network of liver-enriched transcription factors. *Dev. Biol.* 192, pp. 247-257 (1997)
- Lannoy, V.J. et al., Isoforms of hepatocyte nuclear factor-6 differ in DNA-binding properties, contain a bifunctional homeodomain and define the new ONECUT class of homeodomain proteins. *J. Biol. Chem.* 273, pp. 13552-13562 (1998)
- Lemaigre, F.P. et al., Hepatocyte nuclear factor-6, a transcription factor that contains a novel type of homeodomain and a single cut domain. *Proc. Natl. Acad. Sci. USA.* 93, pp. 9460-9464 (1996)
- Rausa, F. et al., The cut-homeodomain transcriptional activator HNF-6 is coexpressed with its target gene HNF-3b in the developing murine liver and pancreas. *Develop. Biol.* 192, pp. 228-246 (1997)
- Samadani, U. & Costa, R.H., The transcriptional activator hepatocyte nuclear factor-6 regulates liver gene expression. *Mol. Cell. Biol.* 16, pp. 6273-6284 (1996)

## CLAIMS

1. A pharmaceutical composition comprising an appropriate pharmaceutical vehicle and an element chosen from among the group consisting of a nucleotide sequence encoding a peptide from the ONECUT family, a vector comprising this nucleotide sequence, the polypeptide sequence encoded by this nucleotide sequence and/or a cell line transformed by the said vector and expressing the peptide from the ONECUT family.
2. The pharmaceutical composition according to claim 1, characterized in that the peptide of the family is HNF-6.
3. The pharmaceutical composition according to claim 1, characterized in that the peptide of the family is OC-2.
4. The pharmaceutical composition according to any one of the previous claims, characterized in that the aforementioned nucleotide and polypeptide sequences are human nucleotide and polypeptide sequences.
5. The pharmaceutical composition according to any one of the previous claims, characterized in that the vector is chosen from among the group consisting of plasmids, viruses, phagemids, lipidic vesicles, particularly cationic vesicles, liposomes or a mixture thereof.
6. The utilization of the pharmaceutical composition according to any one of the previous claims for the preparation of a medication intended for the prevention and/or treatment of type 1 or type 2 diabetes or the conditions associated with diabetes, and to the prevention and/or treatment of cancer, particularly melanoma.
7. The therapeutic procedure for treating the patient, preferably a human patient at risk for developing or suffering from diabetes or cancer, particularly melanoma, characterized in that the pharmaceutical composition according to any one of claims 1 to 3 is administered ex vivo by isolating a bodily fluid or one or more cells from the patient, by treating the said cells or the cells present in this bodily

fluid by a vector of the invention, and by reinjecting the transformed cells into said patient.



### OC-2 Sequence

CCCCGCCCCGCCCCGGGCCCTGATGGACTGAATGAAGGCTGCCTACACCGCCTATCGATGCCTCACCAA 69  
AGACCTAGAACGTGCGCCATGAACCCGGAGCTGACAATGGAAAGTCTGGGCACCTTTGCACGGCGCGCGC 137  
M N P E L T M E S L G T L H G A R 17  
GGCGGCGGCAGTGGCGGGGGCGGCGGG 207  
G G G S G G G G G G G G G G G G G G G G G G P G H E Q 40  
GAGCTGCTGGCCAGCCCCAGCCCCACCACGCGCGCGCGCGCGCGCGCTGGCTCGCTGCGGGGCCCTCCG 276  
E L L A S P S P H H A R R G P R G S L R G P P 63  
CCGCCTCCAACCCCGCACCCAGGAGCTGGGCACCGCGGCGAGCGGCGGCGAGCGGCGGCGTCCGCGCTCGGCC 345  
P P P T A H Q E L G T A A A A A A A A S R S A 86  
ATGGTCACCAGCATGGCCTCGATCCTGGACGGCGGCGACTACCGGCCCGAGCTCTCCATCCCGCTGCAC 414  
M V T S M A S I L D G G D Y R P E L S I P L H 109  
CACGCCATGAGCATGTCTGCGACTCGTCTCCGCTGGCATGGGCATGAGCAACACCTACACCACGCTG 484  
H A M S M S C D S S P P G M G M S N T Y T T L 132  
ACACCGTCCAGCGGCTGCCACCCATCTCCACCGTGTCTGACAGTTCCACACCCCTCACCCGCAACCAC 552  
T P L Q P L P P I S T V S D K P H H P H P H H 155  
CATCCGCACCAACCACCAACCACCAACCACCAAGCGCCTGTCCGGCAACGTCAGCGGCAGCTTCACCCCTC 621  
H P H H H H H H H R Q R L S G N V S G S F T L 178  
ATGCGCGACGAGCGGGGCTCCCGCCATGAACAACCTCTACAGTCCCTACAAGGAGATGCCCCGGCATG 690  
M R D E R G L P A M N N L Y S P Y K E M P G M 201

Figure 1



OC-2 Sequence (CONTINUED)

AGCCAGAGCCTGTCCCCGCTGGCCGCCACGCCGCTGGGCAACGGGCTAGGCGGCCTCCACAACGCGCAG 759  
S Q S L S P L A A T P L G N G L G Q L H N A Q 224  
CAGAGTCTGCCCACTACGGTCCGCCGGGCCACGACAAAATGCTCAGCCCCAACTTCGACGCGCACCAC 828  
Q S L P N Y G P P G H D K M L S P N F D A H H 247  
ACTGCCATGCTGACCCGCGGTGAGCAACACCTGTCCCGCGGCTGGGCACCCACCTGCGGCCATGATG 897  
T A M L T R G E Q H L S R G L G T P P A A M M 270  
TCGCACCTGAACGGCCTGCACCACCCGGGCCACACTCAGTCTCAGGGCGCGGTGCTGGCACCAGTCGC 966  
S H L N G L H H P G H T Q S H G P V L A P S R 293  
GAGCGGCCACCCCTCGTCTCATCGGGCTCGCAGGTGGCCACGTGGGGCCAGCTGGAAGAAATCAACACC 1035  
E R P P S S S S G S Q V A T S G Q L E E I N T 316  
AAAGAGGTGGCCACGCGCATCACAGCGGAGCTGAAGCGCTACAGTATCCCCCAGGCGATCTTTGCGCAG 1104  
K E V A Q R I T A E L K R Y S I P Q A I F A Q 339  
AGGGTGTCTGTCCGGTCTCAGGGGACTCTCTCCGACCTGCTCCGGAATCCAAAACCGTGGAGTAAACTC 1173  
R V L C R S Q G T L S D L L R N P K P W S K L 362  
AAATCTGGCAGGGAGACCTTCCGCGAGATGTGGAAGTGGCTTCAGGAGCCCGAGTTCCAGCGCATGTCC 1242  
K S G R E T F R R M W K W L Q E P E F Q R M S 385  
GCCTTACGCTGGCAGCGTGCAAACGCAAGAGCAAGAACAAACAAAGACAGGAACAATCCAGAGAG 1311  
A L R L A A C K R K E Q E P N K D R N N S Q K 408  
AAGTCCCGCCTGGTGTTCACCTGACCTCCAACGCCGAACACTCTTCGCCATCTTCAAGGAGAACAAACGC 1380  
K S R L V F T D L Q R R T L F A I F K E N K R 431  
CCGTCAAAGGAGATGCAGATCACCATTTCCAGCAGCTGGGCCCTGGAGCTCACAACCGTCAGCAACTTC 1449  
P S K E M Q I T I S Q Q L G L E L T T V S N F 454  
TTCATGAACGCCCCGCGCGCAGCCTGGAGAAGTGGCAAGACGATCTGAGCACAGGGGGCTCCTCGTCC 1518  
F M N A R R R S L E K W Q D D L S T G G S S S 477  
ACCTCCAGCAGTGTACCAAAGCATGATGGAAGGACTCTCACTTGGGCACAAGTCACCTCCAAATGAGG 1587  
T S S T C T K A

Figure 1b



PHARMACEUTICAL COMPOSITION FOR TREATING OR  
PREVENTING ...  
Rousseu, et al.  
Appl. No.: 09 763,535 Atty Docket: DECLE26.001APC

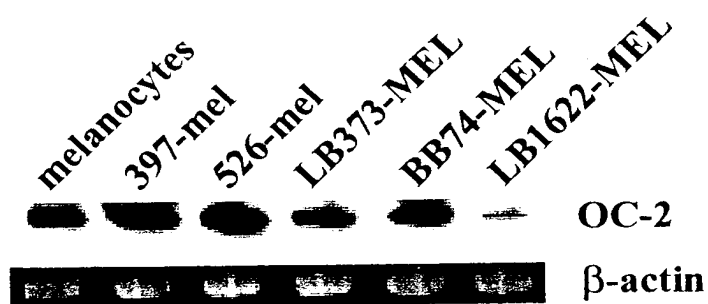


FIG. 1





PHARMACEUTICAL COMPOSITION FOR TREATING OR PREVENTING

Rousseau, et al

Appl. No. : 09 763,535 Atty Docket : DECLE26.001APC

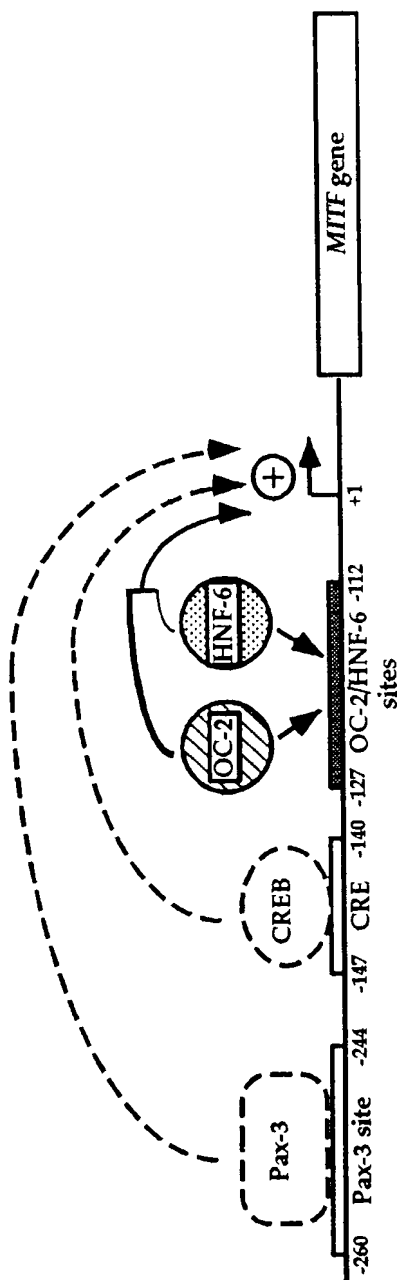


FIG. 2